JOURNAL

OF THE

New York Entomological Society

Vol. LVII June, 1949 No. 2

PHYSIOLOGICAL EFFECTS OF INDUCED HEMORRHAGE IN JAPANESE BEETLE LARVÆ

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In the course of studying various aspects of Japanese beetle (*Popillia japonica* Newm.) larvæ infected with *Bacillus popilliæ* Dutky (Beard, 1945) it was observed that blood (hemolymph) samples could be taken repeatedly from individual grubs without apparent ill effects. Since the size of the samples was relatively large on occasion, rapid replacement of blood was indicated. Confirmation of this was the primary objective of the observations reported here.

These investigations were made upon third instar larvæ of the Japanese beetle, incubated in soil with sprouting grass seed for food and maintained at a constant temperature of 80°F.

In interpreting the effects of loss of blood induced by puncturing the integument, it is important to know the normal blood volume present in an individual grub. The total blood volume of insects is difficult to determine with accuracy because of unsatisfactory techniques. Approximations can be made, however, using several methods (Richardson *et al,* 1931; Yeager and Tauber, 1932; Yeager and Munson, in press). As much as 29 per cent of the body weight of a third instar Japanese beetle grub has been lost at a single bleeding, so the total blood volume must exceed this figure at least in some individuals.

The method of Richardson *et al.*, (1931) for determining blood volume, in which the animal is cut open and the blood taken up with filter paper, yielded values ranging from 25.1 per cent to 40.8 per cent of the body weight, the mean of ten determinations being 31.9 per cent . Since these figures are little more than can be obtained by draining blood from punctured grubs killed by immersion in water heated to 60° C., the values are undoubtedly low. The difficulties with this method lie in the facts that blood in the legs, head, and other inaccessible places, is not removed, and on the other hand, as blood is removed, the fat body and other soft, loosely attached tissues may be taken out inadvertently.

Although the dye dilution method of determining blood volume is the simplest and is suitable for some insects (Yeager and Munson, *loc. cit.*), it is not usable for Japanese beetle larvæ. The dyes most useful for this purpose—Amaranth or Ponceau 3 R—when injected into beetle grubs, are not circulated promptly, but appear to be taken up locally by other tissues. Moreover the injection irritates the insect to the extent of inducing the grub to bite itself, thus causing hemorrhage. Attempts to artificially circulate the dye by gently massaging the grub while under carbon dioxide anaesthesia, have not been successful because the gut usually ruptured with such treatment.

The injection of chloride (as NaCl) and its subsequent determination after dilution by the blood likewise did not prove feasible. With this technique the chief difficulty is in the lack of a precise end-point when the chloride is titrated with silver nitrate. Neither the method of Yeager and Munson (*loc. cit.*) nor the adsorption indicator method described by King (1947) yielded reliable results with this species of insect.

Another technique, which is peculiarly adapted to determining the blood volume in Japanese beetle larvæ, has several advantages but certain technical disadvantages. This method depends upon the presence of bacterial spores (*Bacillus popilliæ*) in the blood. Infected individuals can be found in the field or infection can be induced readily by rearing grubs in inoculated soil. The technique assumes that the spores are resident only in the blood and that their distribution in the blood is uniform. Both of these as-

sumptions are entirely reasonable in view of observations reported previously (Beard, 1945). The results obtained, however, must be expressed in terms of infected individuals, for at present it is not known if the presence of the bacteria otherwise affects the blood volume of the insects. The method, then, is to determine the number of spores in a given quantity of blood and also the total number of spores present in the grub. From these data the blood volume can be calculated simply. The concentration of spores in the blood is determined by making suitable dilution and counting with a hemocytometer. The total number of spores present is found by macerating the grub, suspending the brei in water, and counting the spores in representative samples, using the counting chamber. The spores have a characteristic shape and usually can be distinguished from the other particles in the suspension. The procedures involved are relatively simple, but two factors make for inaccuracy. One is that the spores are so numerous that great dilution is required for counting. This means that large sampling errors may occur. The other is that even though the spore is characteristic in shape, it cannot always be distinguished with certainty from other bits of tissue of the macerated grubs. A different type of counting than is usually employed, however, tends to reduce the personal error and makes possible a statistical check on the reliability of the sampling method, (Bliss, 1948). Instead of making only a total numerical count of the spores present within a given number of squares of a counting chamber, the number of squares containing 0, 1, 2, 3, . . . n spores is recorded for a total of 80 squares in each of four fields sampled separately. Agreement with Poisson distribution then serves to check on the randomness of the sample and subsamples. Bliss (*loc. cit.*) has described a method for using a truncated Poisson distribution, in which the squares of the counting chambers would be recorded in terms of those containing 0, 1, 2, 3, and 4 or more spores. While this simplifies somewhat the actual recording of data, it seems more satisfactory to record the entire series for greater precision, and in some respects, for greater ease of both execution and calculation. As might be expected, this technique demonstrated that the determination of the number of spores per unit volume of blood was more accurate

than the determination of the total number of spores present in the grub. The technique can be used, however, to yield figures in which some confidence can be placed. The time required for making the determinations and calculations was great enough that, when a simpler method, described below, presented itself, no more determinations were made than were required to establish the validity of the technique.

A third dilution technique differing from those mentioned above was employed successfully by taking advantage of the spectrographic method of analysis. A known amount (7 lambda[1]) of a 3 per cent solution of manganese chloride was injected into a beetle grub of known weight anaesthetized with carbon dioxide. After a definite time interval, the grub was killed by immersion in water heated to 60° C. to inhibit blood coagulation, and a sample of blood (20 lambda) was withdrawn and absorbed on filter paper. The filter paper was then ashed, the residue taken up in 1 ml. of water, and samples were tested spectrographically[2] for manganese. This element was chosen because it is a normal constituent of the blood of this insect and because its spectrographic lines are very sensitive. Blanks and standards were prepared simultaneously by adding a known volume of $MnCl_2$ to known volumes of pooled blood. Samples (20 lambda) of these were absorbed on filter paper and analyzed in the same way as the other blood samples. By this means the normal manganese of the blood was taken into account without further correction. The standards prepared yielded reliable and reproduceable curves with which the unknowns could be compared and calculated in terms of blood volume. Determinations were made on three groups of nine grubs each, samples being taken after killing the grubs five, 10, and 20 minutes after injection. This was done because the time required for circulation, and hence good distribution, of the injected material was not known. Too short a time interval would be expected to give erratic results, whereas too great a time interval might permit the injected material to be taken up by other tissues, excreted, or possibly even induce dilution of the blood if the osmotic balance

[1] (= .007 ml.)

[2] The writer gratefully acknowledges the assistance given by Mr. W. T. Mathis in designing this technique and executing the spectrographic analyses.

was appreciably upset by the $MnCl_2$. The volume of blood found for each grub was converted to volumes per cent based on body weight, the means being as follows:

TABLE 1

	Mean blood vol. per cent	Standard error
Grubs killed 5 min. after injection	38.91	1.62
Grubs killed 10 min. after injection	42.85	2.03
Grubs killed 20 min. after injection	40.91	2.34
All determinations	40.89	1.16

Although the lower figure might suggest that five minutes was not sufficient time for the $MnCl_2$ to circulate, the difference between the mean of this group and that of grubs killed 10 minutes after injection is not statistically significant. It would appear, then, that the mean of all determinations could serve as a reasonable figure for the average blood volume of a Japanese beetle larva.

Although the hemolymph of the Japanese beetle grub coagulates promptly upon exuding from a wound, this coagulation has little effect in reducing hemorrhage, chiefly because there is an immediate and copious outflow of blood when the integument is punctured. This is borne out by the following experiment. Each of ten grubs was pricked with a needle and as the blood flowed out it was taken up with absorbent paper before it coagulated. Another series of ten grubs was similarly pricked, but the blood was allowed to coagulate before it was wiped off. Weight measurements before and after bleeding indicated the amount of hemorrhage. In the former group the loss of blood per grub ranged from 8.86 per cent body weight to 23.89 per cent with a mean of 15.74 per cent. Among the grubs whose blood was allowed to coagulate, the blood loss ranged from 5.72 per cent to 25.44 per cent, with a mean of 14.08 per cent. These differences are not significant. Bleeding tends to be less in quiet individuals which maintain good muscle tonus. The wounds in flaccid individuals do not seem to close as promptly as in others, and those

highly active tend to "pump out" blood by their body movements. It has also been observed that the activity of grubs when removed from soil may open up old wounds, indicating that coagulation has not formed an efficient plug before tissue growth has healed the wound.

The maximum amount of blood a given grub can lose at one time without fatal results is, of course, impossible to determine. Among different grubs the amount that flows freely from an induced wound, without external pressure being applied, varies within broad limits. It has already been noted that in one instance 29 per cent of the body weight was lost, and among 200 grubs punctured with a needle, the loss of blood ranged from 1.3 milligrams to 59.7 milligrams, or from 0.9 per cent to 22.5 per cent of the body weight. Several observed grubs have survived losses of blood in excess of 20 per cent of their body weight, indicating that possibly as much as 50 per cent of their total blood volume might be lost at one time without fatal effects.

The effect of a single bleeding upon subsequent growth as measured by body weight was observed by wounding a series of grubs and comparing the body weights with those of uninjured grubs incubated under the same conditions. If the initial weight is taken as a point of reference, the uninjured larvæ followed the more or less expected sigmoid growth curve. Among the punctured grubs, the loss of blood, of course, resulted in an immediate reduction in body weight. This was followed by a slight further reduction in weight which may have been associated with interrupted feeding activity, as it has been observed that normal grubs when taken from abundant to restricted pasture may similarly lose weight temporarily. Although the weaker grubs continue to lose weight and eventually die, the tendency is toward a recovery of the original weight within three days after injury and a continued increase in body weight at a rate depending upon the vigor of the individual. The most vigorous individuals gain weight more rapidly than the uninjured controls, but the general tendency is to parallel the growth rate of the controls with a definite time lag. Depending upon the amount of blood lost, pupation may be delayed two or even three weeks.

Similar observations were made on the effect of repeated hem-

orrhages in terms of the body weight changes during a period of two weeks. Groups of grubs were punctured one, two or three times each week, or two, four or six times during the period of observation. Individual weight records were kept and the amount of blood lost was noted. The data obtained from the grubs surviving treatment are summarized in Table II, which indicates the changes in body weight based upon the means within each group and expressed in terms of the per cent of the initial weight. The net increase represents the body weight changes exclusive of the blood removed, whereas the gross increase includes the weight of the blood lost as this is tissue formed by the insect, but withdrawn from it. Data on one individual are included to indicate an extreme case.

TABLE 2

Mean Changes in Body Weight

Number of woundings	Number of insects	Per cent of initial weight		
		Blood lost	Net increase body weight	Gross increase body weight
0	16	.....	47	47
2	20	25	38	63
4	22	45	28	73
6	15	51	14	65
6	1	68	−17	51

Although these summary data obscure the individual variation, it is apparent that the greater the loss of blood, the slower is the rate of increase of net body weight. In other words, blood replacement is made at the expense of other tissue formation. It is significant, however, that 50 per cent or more of the body weight can be lost as blood in a two week period and there still be an increase in net body weight. This means that the volume of blood lost probably more than equalled the normal blood volume present at one time, considering the normal blood volume to be the 40.89 per cent mentioned above. In an extreme case of the one individual indicated, which lost 68 per cent of its body weight as blood in six hemorrhages, replacement of this loss was made at the expense of maintaining its initial body weight. Even so, if

this blood is included as tissue formation, the gross increase is more than equal that of the mean of the uninjured controls.

As might be expected not all grubs can withstand the drastic treatment imposed upon them in the above test, and the mortality reflects the severity of hemorrhage. This is evidenced by the mortality figures of three series of observations involving a total of 160 grubs—40 in each of the groups indicated below.

TABLE 3

Number of grubs	Number of hemorrhages	Per cent mortality at end of two weeks
40	0	17.5
40	2	37.5
40	4	35.0
40	6	52.5

In this series the higher value of 37.5 per cent over 35.0 probably has no significance.

It is doubtful if infection was responsible for any of this increase in mortality attending wounding. Although fatal infection invariably occurs if the gut is punctured, it has rarely been observed that infection from soil bacteria has resulted from wounding. Beard (1945) has given experimental evidence that preliminary wounding does not increase the infection rate among grubs exposed to heavy spore concentration of *Bacillus popilliæ,* a bacteriemic parasite of this insect.

Although the above experiments suggest a fairly rapid quantitative replacement of blood following hemorrhage, a somewhat more direct estimate of the time required for the blood volume to return to normal can be made. Actual volume determinations using the manganese dilution technique would of course be the most precise, but a simpler method can be used for comparative purposes in determining the time factor, if not the actual volume relationships. If grubs are heat-fixed to prevent blood coagulation, it is found that considerably more blood can be drawn from normal grubs than from grubs that have been wounded previously. Accordingly, by determining the amount of blood that can be drawn from wounded grubs at various times after hem-

orrhage in comparison with that from intact controls, the approach to normal can be approximated. In such a test, the wounded grubs yielded 67 per cent of that from uninjured controls seven hours after hemorrhage, 88 per cent 24 hours after hemorrhage, and 91 per cent after 48 hours. Of course these figures definitely exaggerate the difference in terms of the true values because of the residual blood that cannot be drained. Although the return to normal is probably more or less asymptotic, these data tend to support the impression gained in the above experiments that normal blood volume is essentially restored within two to three days after hemorrhage.

Some consideration was given to the qualitative effects of induced hemorrhage.

Specific gavity determinations were made on samples of blood taken from grubs seven, 24, and 48 hours after wounding, using a falling drop technique essentially that of Barbour and Hamilton (1926). Similar determinations were made on samples taken from intact grubs handled under the same environmental conditions. Successive samples could not, of course, be taken from the same grub, so for each time interval, groups of 5 larvæ each were tested independently. The mean specific gravity for each group was found to be as follows:

TABLE 4

	Mean loss of blood (mg/gm body weight)	Specific gravity Hours after hemorrhage		
		7	24	48
Wounded grubs	45.6	1.0294		
	95.2		1.0308	
	61.4			1.0311
Intact controls	0.0	1.0408	1.0346	1.0338

As compared with the controls, the specific gravity of blood from the test grubs was significantly less among the samples taken seven hours after hemorrhage but, as time went on, it gradually approached the normal control, the differences being not statistically significant at the 24 and 48 hour intervals. It will be noted, however, that the reduction of the difference between the

groups is due less to an increase in specific gravity of the test insects than it is to a decrease in the specific gravity of the blood from the control grubs. The reason for the latter is not known, but it may be associated with the change in pasture, since all grubs were removed from a common rearing container to individual containers for each group with a replenished source of food. The fact that the second group of wounded grubs lost an appreciably greater amount of blood than the other groups does not seem to affect the recovery trend.

Spectrographic analyses were made* of pooled samples of blood taken from grubs seven, 24 and 48 hours after hemorrhage induced by wounding. Similar analysis was made of blood from comparable controls, duplicates being run of both series. The spectrographs of all samples were similar in most respects and, while certain slight variations appeared, these could not be attributed to the effect of loss of blood prior to sampling. The lines representing aluminum, copper, magnesium, and phosphorus were remarkably uniform. Those of sodium, calcium, manganese, and iron showed more variation, but the control samples varied as much among themselves as with the samples from bled grubs. One notable difference appeared, however, in both of the duplicate blood samples taken seven hours after hemorrhage. This was a difference in the potassium lines, indicating a definite reduction in the blood K, which was not apparent in the samples taken 24 and 48 hours after injury. It would seem that chemical balance is restored within 24 hours, but following bleeding there is a depletion of blood K due either to a selective withdrawal of K by other tissues, or what is more likely in view of the reduction in blood density, to a slower replacement of K than of the other common elements at the time the internal water balance is being restored.

From previous observations, both published and unpublished, it is known that the blood cell counts of Japanese beetle larvæ may vary within wide limits even under apparently normal conditions. It is not surprising then, to find that blood cell counts taken approximately six hours after induced hemorrhage do not differ significantly from those of grubs not wounded, as judged

* These analyses were made through the kindness of Mr. W. T. Mathis.

by four determinations in each group. The data obtained are as follows:

TABLE 5

Blood cells per mm³ in

	Wounded grubs	Intact grubs
	6,200	10,450
	8,600	14,825
	19,600	19,300
	34,950	33,100
Mean	17,337	19,419

Although the accumulation of a larger mass of data might demonstrate a statistically significant reduction in the number of blood cells in wounded grubs, it seems apparent from these few figures that there is no very striking difference and additional counts do not seem called for in an attempt to prove a difference.

Concluding from a single test series, there is no apparent change in the blood pH following hemorrhage, as measured by a quinhydrone electrode designed to measure pH of single drops of fluid. Six hours after hemorrhage was induced by puncturing the integument, determinations were made on the blood of each of five wounded grubs and of five intact grubs as controls. Although too much confidence should not be placed in the results because of the technique employed, the variation among the grubs within a group was great enough to indicate no statistical difference between the groups. The pH of normal blood exposed to air may be taken to be approximately 7.2.

Discussion: From the data presented, it is obvious that although loss of hemolymph is not uniformly tolerated, surprisingly large volumes may be drained from a grub, particularly if repeated wounds are inflicted. Comparatively little is known about hemotopoiesis in insects, although it is generally believed that new blood cells are formed from those in circulation. The wide variation in numbers of hematocytes normally found make it difficult to learn much about blood replacement from them, but in view of the various functions of the blood, perhaps the plasma relationships are more important. The diminished spe-

cific gravity of the residual blood following wounding suggests that other tissue fluids are poured into the circulating fluid which is thereby diluted, but this cannot be adequate in meeting the loss because the total blood volume remains subnormal for a period of two to three days. Apparently this is not restored until ingestion of succulent food and the accumulation of metabolic water make up the loss. There also appears to be a rapid mobilizing of the common inorganic constituents with the exception of potassium, the replacement of which is delayed. Undoubtedly the picture would be clearer if quantitative determinations had been made of the organic constituents of the blood, particularly those of glucose, glycogen, and amino acids. In any case it seems certain that when tissue fluid replaces that lost from the circulation, various constituents are contributed more or less independently and at different rates before the "normal" balance is restored.

SUMMARY: The mean volume of blood in a third instar Japanese beetle larva was estimated to be approximately 40 volumes per cent body weight, as determined by a manganese dilution technique using spectrographic analysis.

Coagulation has little effect in reducing hemorrhage induced by puncturing the integument, and in some individuals an estimated 50 per cent or more of the blood volume can be lost at one time without fatal results. Hemorrhage results in a loss of body weight greater than the weight of the blood lost, but this tends to be restored within two to three days following injury. Thereafter body weight increases tend to parallel the growth rate of uninjured controls, but with a definite time lag. Pupation may be delayed two to three weeks, depending upon the amount of blood lost. Repeated hemorrhage causes mortality in direct relation to the extent of bleeding, but among the survivors of such treatment, 50 per cent or more of the body weight can be lost as blood in a two week period and there still be an increase in net body weight.

Quantitatively, blood replacement approaches completion two to three days following hemorrhage. Qualitatively, the specific gravity of the blood becomes less following hemorrhage, but approaches the normal after 24 hours. Similarly there is a de-

crease in blood potassium following loss of blood, but this is restored within 24 hours. Other elements tested appear to be unaffected by this type of injury. The pH of the blood and the number of blood cells per unit volume appear to be relatively unchanged as a result of bleeding.

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